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High water solubility and fold in amphipols of proteins with large hydrophobic regions: Oleosins and caleosin from seed lipid bodies

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ABSTRACT

Seed lipid bodies constitute natural emulsions stabilized by specialized integral membrane proteins, among which the most abundant are oleosins, followed by the calcium binding caleosin. These proteins exhibit a triblock structure, with a highly hydrophobic central region comprising up to 71 residues. Little is known on their three-dimensional structure. Here we report the solubilization of caleosin and of two oleosins in aqueous solution, using various detergents or original amphiphilic polymers, amphipols. All three proteins, insoluble in water buffers, were maintained soluble either by anionic detergents or amphipols. Neutral detergents were ineffective. In complex with amphipols the oleosins and caleosin contain more beta and less alpha secondary structures than in the SDS detergent, as evaluated by synchrotron radiation circular dichroism. These are the first reported structural results on lipid bodies proteins maintained in solution with amphipols, a promising alternative to notoriously denaturing detergents.

1. Introduction

Highly hydrophobic proteins, including integral membrane proteins (IMPs) are prone to severe aggregation in water. Their handling in aqueous solution is a critical issue, not only in fundamental biochemistry research, but also from a practical perspective. IMPs inserted in the cell membrane phospholipid bilayer are the targets of the majority of commercialized drugs [1]. Besides, structural membrane proteins found in the phospholipid monolayer of lipid storage organelles (lipid bodies: LBs, also called oil bodies, lipid droplets or oleosomes) are also related to health issues. Human low density lipoproteins are associated with coronary heart disease risk and seed oil body proteins to allergies [2]. Due to their role in organelles stabilization, the latter proteins have an important impact on oil extraction from seed LBs of economically important oleaginous plants (rapeseed, soy, sesame…).

In oleaginous plants, neutral lipids are stored into specialized organelles, with diameter ranging from 0.2 to 3 μm. These LBs represent the source of energy for seeds. Plant LB proteins fall into two types: i) structural proteins, the most abundant, mainly represented by oleosins. In *Arabidopsis thaliana*, five seed-specific oleosins (S1 to S5) with molecular mass comprised between 14 and 21 kDa have been detected [3]; ii) minor proteins such as caleosin, a calcium binding protein [4], stereoleosin, an enzyme using hydroxysteroids as substrates *in vitro* [5] and lipases [6]. LB integral proteins are important in seed tissue for controlling oil body structure and lipid accumulation [7]. Thus, oleosomes remain small in size, allowing a quick mobilization of lipids for germination, a period of active metabolism.

The organization of IMPs inserted into a monolayer of phospholipids is poorly known. Thus, few data concern the structure of oleosins, even less data being available for caleosin. Caleosin and oleosins, despite low sequence identity (26%), share a similar amphiphilic triblock architecture. The central region is highly hydrophobic. In the case of oleosins it is the longest hydrophobic region (71 residues for the two oleosins studied in this work, S3 and S5) known to occur in natural proteins. This is in accordance with its insertion into a phospholipid monolayer, and probably into the lipid core packed by this monolayer. This central region comprises three conserved prolines forming a characteristic proline knot motif, which is involved in protein targeting to lipid bodies, and is flanked with polar N- and C-termini of variable lengths [8]. Upon calcium binding, the lipid bodies' interfacial behaviour as well as caleosin's interfacial properties are strongly modified [9], making caleosin an interesting target for understanding lipid bodies stability. Caleosin central hydrophobic region is significantly shorter (41 residues) than

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that from oleosins, which makes caleosin (MW 28 kDa) a more hydrophilic protein. Oleosin S5 is the shortest member (14.9 kDa) of the oleosin family, due to shorter N and C termini. It is therefore the most hydrophobic oleosin. Oleosin S3 (18.6 kDa), the most abundant oleosin in *A. thaliana* seeds, with N- and C-termini longer than those of S5, is less hydrophobic. S3 and S5 share 63% sequence identity. There are very few solved high-resolution structures of full-length integral proteins from LBs. They mainly belong to apolipoproteins (for instance human apo A-I [10] or locust apolipophorin 3 [11]). For triblock IMPs from lipid bodies, there is no known high-resolution structure yet.

The difficulty in handling and purifying IMPs derives directly from their amphiphilic structures, which display regions adapted to interact with the lipid membrane and other regions protruding toward the aqueous media. In order to achieve structural studies of such proteins, IMPs have to be solubilized in aqueous solutions in conditions that prevent aggregation between their hydrophobic regions. This has been traditionally achieved by using small molecular surfactants added in a concentration close to their critical micellar concentration (CMC). These surfactants (called detergents) cooperatively assemble on the hydrophobic regions of the proteins, maintaining solubility as long as the total detergent concentration is higher than its CMC. However, the dilution of the detergent below its CMC usually triggers the aggregation of the IMPs, whereas high detergent concentrations may denature the proteins. In addition, the radius of detergents micelles is matching with the height of transmembrane domains from the plasma membrane proteins, but might be less adapted to protect longer hydrophobic domains like those of oleosins and caleosin that probably protrudes into the lipid core of the LBs.

Different authors extracted oleosins and caleosin from seeds with organic solvents [12] or tried to solubilize their recombinant forms using alcohols [13], urea [14] or SDS [15], and recently various detergents [16]. However, none of these authors used stringent enough criteria to measure solubility, centrifugation when used being far below the conditions used in the present study (200 000 *g* ultracentrifugation). The use of milder detergents or polymeric amphiphiles called amphipols [17] is expected to provide better conditions for structural studies.

Amphipols (APols), like detergents, protect the hydrophobic domains of IMPs from contact with water. These polymeric surfactants can maintain soluble most membrane proteins found in phospholipids bilayers [18], irrespective of their secondary structure. When APols are used instead of detergents, the stability upon the dilution of transmembrane proteins/amphiphile complexes is significantly enhanced [19]. So far, APols have not been assayed with IMPs from the lipid bodies. Conventional APols have a random distribution of octyl hydrophobes in their chain. As molecular detergents, they form micelles with radii typically below 5 nm. In this study, we used three polymers (quoted A8-35R, A12-60R and A12-80R: R stands for random) as representative of random APols (Fig. 1, and Table 1). In addition, we considered other APols that assemble in larger assemblies. The integration level of octyl hydrophobes in these original polymers (quoted A12-80B, A12-80B1 and A12-80B2: B for blocky) is essentially the same as in A8-35R, but their distribution in the chain is multiblock instead of random [20]. We studied the efficiency of these polymers and detergents (either charged or neutral) to solubilize caleosin and S5 oleosin. The size of APols, protein/APol, and protein/detergent complexes were characterized by X-ray and light scattering. Secondary structure content of LB proteins in different surfactant environments was determined by synchrotron radiation circular dichroism (SRCD).

2. Materials and methods

2.1. Amphipol synthesis

Conventional amphipols (APols) are typically obtained by radical copolymerization of hydrophilic and hydrophobic monomers [21], or modification of commercially available hydrophilic parent chain [22], which is a poly(acrylic) acid in the case of the most popular APol A8-35R. Both approaches result in macromolecules with relatively high dispersity in length (polydispersity index Ip ~1.7) and statistic distributions of hydrophobes. To obtain APols with lower dispersity, we synthesized a parent poly(ter-butyl methacrylate) by controlled radical polymerization (atom transfer radical polymerization ATRP, Ip<1.2) as described in Ref. [20]. Following extensive acidolysis, the parent chain was post-modified by coupling with octylamine either in homogeneous solution of N-methylpyrrolidone (to obtain random copolymers) or in aqueous micellar solution (yielding blocky polymers). Polyacrylic acids with low (home made) and high Ip (Sigma chem., Mw 5000 g/mol, Ip ~1.7) were modified by the same procedure. In the case of blocky polymers, modifications were carried out in micellar solutions of sodium dodecyl sulfate (SDS) [20]. SDS was removed by precipitation in 1 M KCl and dialysis against water (Spectrapor membranes, Slide-A-Lyzer, MWCO 3500). The presence of residual SDS was detected by NMR in some samples, even after 2-day long dialysis of the polymer against water. We checked that the SDS in polymer solution did not modify by more than 10% the radii and MW of polymer self-assemblies [20]. We used only those samples with the lowest SDS:polymer fraction (≤0.06 g/g). The maximal residual SDS amount found in blocky APols (0.06 g/g) is not responsible for protein solubilization: as an example, 1 g A12-80B1/g Clo results in 78% Clo solubilization; this corresponds to 0.06 g SDS/g Clo, which accounts for only 12% solubilization (Fig. 3 A and B). The composition and structural parameters of polymers are given in Table 1. A12-60R, A12-80R, A12-80B, A12-80B1 and A12-80B2 contain a similar average density of octylacrylamide as A8-35R. They differ from A8-35R in that they contain a sodium methacrylate hydrophilic moiety instead of sodium acrylate, and no isopropyl side groups (Fig. 1). Modification of the parent chain in homogeneous conditions yielded random copolymers similar to the most used APol A8-35R (a random terpolymer of octylacrylamide, isopropylacrylamide and sodium acrylate); modification in micellar aqueous dispersion yielded multi-blocky distributions of the hydrophobes.

2.2. Transmission electron microscopy (TEM) and Cryo-TEM on amphipols

Cryo-TEM experiments were carried out on a FEI CM120 electron microscope equipped with a LaB6 filament and operating at 100 kV.

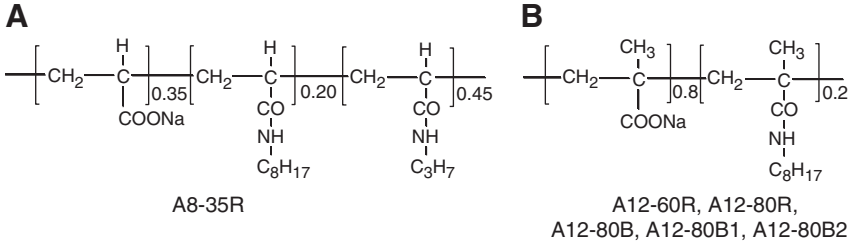

Fig. 1. Chemical structure of amphipols used in this study. (A) Polyacrylate-based polymer A8-35R, random distribution of side chains. (B) Polymethacrylate-based polymers with random (A12-60R and A12-80R) or blocky (A12-80B, A12-80B1, and A12-80B2) distribution of side chains. Molar fraction distribution is indicated in lower case.